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(57) Abstract

DNA encoding modified, secretable erythropoietin proteins whose ability to regulate the growth and differentiation of red blood cell progenitors are different from the wildtype recombinant erythropoietin and to methods of modifying or altering the regulating activity of a secretable erythropoietin and using modified secretable erythropoietin proteins.

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RECOMBINANT HUMAN ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY

Description

Background

The glycoprotein hormone erythropoietin regulates the growth and differentiation of red blood cell (erythrocyte) progenitors. The hormone is produced in the fetal liver and adult kidney. Erythropoietin induces proliferation and differentiation of red blood cell progenitors through interaction with receptors on the surface of erythroid precursor cells.

Several approaches have been employed to identify those features of the protein that are relevant to its structure and function. Examination of the homologies among the amino acid sequences of erythropoietin proteins of various species has demonstrated several highly conserved regions (McDonald, J.D., et al., Mol. Cell. Biol. 6: 842-848 (1986)).

Oligonucleotide-directed mutagenesis has been used to prepare structural mutants of erythropoietin, lacking specific sites for glycosylation. Studies indicate that N-linked carbohydrates are important for proper biosynthesis and/or secretion of erythropoietin. These studies also show that glycosylation is important for in vivo, but not in vitro, biological activity. (Dube, S., et al., J. Biol. Chem. 263:17516-17521 (1988); Yamaguchi, K., et al., J. Biol. Chem. 266:20434-20439 (1991); Higuchi, M., et al., J. Biol. Chem. 267:7703-7709 (1992)).

Studies with monoclonal anti-peptide antibodies have shown that the amino terminus and the carboxy-terminal region (amino acids 152-166) of erythropoietin may be involved with biological activity. It has also been

demonstrated that antibodies to amino acids 99-119 and 111-129 block the hormone's biological activity, apparently by binding to two distinct non-overlapping domains (99-110 and 120-129). (Sytkowski, A.J. and Donahue, K. A., J. Biol. Chem. 262:1161-1165 (1987)). Thus, it was hypothesized that amino acids 99-129 were important in the formation of a functional region involved in receptor recognition, either through forming a necessary component of the protein's tertiary structure or through direct participation in receptor binding, or both.

Preliminary experiments suggested that alterations in localized secondary structure within the 99-129 region resulted in inactivation of erythropoietin. Therefore, a possible structural role for amino acids 99-129 has been postulated. Recently, a series of experiments indicated that amino acids 99-110 (Domain 1) play a critical role in establishing the biologically active conformation of human erythropoietin. (Chern, Y., et al., Eur. J. Biochem. 202:225-229 (1991)).

These Domain 1 mutants, in which a group of three amino acids was deleted and replaced by two different amino acids, were found to be biologically inactive. Furthermore, these mutations in Domain 1 inhibited the secretion of the mutant erythropoietin into cell culture medium. (Chern, Y., et al., Eur. J. Biochem. 202:225-229 (1991)). Inhibition of secretion in mammalian cells is consistent with a profound structural change of the polypeptide hormone. Profound structural changes could significantly affect the ability of the hormone to interact with its cognate receptor. Thus, these mutant erythropoietin polypeptides are not suitable for elucidating the structure/function relationship that exists between erythropoietin and its cellular receptor. Nor are these mutants suitable erythropoietin antagonists for use, for example, in therapeutic treatment of

polycythemias, or over production of erythropoietin. Thus, it would be beneficial to precisely determine which amino acids are critical to the erythropoietin polypeptide to maintain a stable, biologically active conformation which retains its secretable properties and its ability to bind to the erythropoietin receptor.

Moreover, the precise determination of critical amino acid residues would be useful to alter the biological activity of erythropoietin, either decreasing or increasing one or more biological properties of the protein.

Summary of the Invention

The present invention relates to DNA encoding mutated erythropoietin proteins which have altered biological activity, yet retain their secretable properties (i.e., secretable erythropoietin proteins). That is, the present invention relates to DNA encoding secretable erythropoietin proteins which have at least one amino acid residue in Domain 1 which differs from the amino acid residue present in the corresponding position of wildtype erythropoietin and which have altered ability to regulate the growth and differentiation of red blood cell progenitors. Domain 1 of the mutants described herein refers to the amino acids which correspond to amino acids 99-110 (SEQ ID NO: 1) of the wildtype recombinant erythropoietin. Altered ability is defined as ability different from that of the wildtype recombinant erythropoietin ability to regulate the growth and differentiation of red blood cell progenitors.

The present invention also relates to the modified secretable mutant erythropoietin proteins encoded by the DNA described above. These modified secretable erythropoietin proteins have altered biological activities. For example, the modified secretable mutant

erythropoietin may have decreased biological ability to regulate growth and differentiation of red blood cell progenitor cells. Alternately, a modified secretable mutant erythropoietin protein described herein may exhibit increased heat stability.

The present invention also relates to methods of modifying or altering the regulating activity of a secretable erythropoietin. Such erythropoietin proteins include at least one amino acid residue different from the amino acid present at the corresponding position in Domain 1 in the wildtype erythropoietin and are referred to as modified secretable human recombinant erythropoietin proteins having altered ability (i.e., decreasing or enhancing ability) to regulate the growth and differentiation of red blood cell progenitors. It is important to note that the ability of erythropoietin to regulate growth and differentiation of red blood cell progenitors depends on the ability of erythropoietin to bind to its cellular receptor. The mutant erythropoietin proteins described herein retain their secretable properties, thus indicating that these mutants also retain their biological conformation and the ability to interact with the erythropoietin receptor.

In particular, this invention relates to modified secretable erythropoietin proteins in which the amino acid sequence of secretable human recombinant erythropoietin is altered at a selected site, or sites, in such a manner that the resulting erythropoietin protein has regulatory ability less than that of the corresponding (unaltered, or wildtype) human recombinant erythropoietin protein (i.e., decreased activity) or regulatory ability greater than that of the corresponding (unaltered, or wildtype) human recombinant erythropoietin protein (i.e., increased activity). Such proteins, which regulate red blood cell growth and differentiation to a lesser or greater extent

than wildtype recombinant erythropoietin, are referred to, respectively, as modified secretable human recombinant erythropoietin proteins with decreased regulating ability and modified secretable human recombinant erythropoietin proteins with enhanced regulating ability.

Human recombinant erythropoietin proteins with altered regulating activity differ from the wildtype human recombinant erythropoietin protein in that the amino acid sequences of the erythropoietin proteins with altered activity are different from the amino acid sequence of the wildtype protein at a site, or sites, found to be critical for the growth and differentiation of red blood cell progenitors. Alteration, as used herein, includes substitution of a different amino acid, as well as deletion or addition of an amino acid.

Until the present invention, mutations within the erythropoietin sequence which result in the alteration of biological activity have also frequently resulted in a concurrent loss of secretability. This loss of secretability is consistent with a loss of structural integrity. (Boissel, J-P. and Bunn, H. F., "The Biology of Hematopoiesis", pp. 227-232, John Wiley and Sons, New York (1989)). Now, the sites critical to the maintenance of a stable, biologically active conformation have been identified by means of oligonucleotide-directed mutagenesis and have been found to occur in Domain 1 (amino acids 99-110) (SEQ ID NO: 1) of human recombinant erythropoietin. Modifications of the wildtype erythropoietin have been made and the encoded erythropoietin proteins have been expressed. The resulting mutant erythropoietin proteins have been shown to have altered erythropoietin regulating activity, as demonstrated in the art-recognized bioassay of Krystal, G., Exp. Hematol. 11:649-660 (1983). Activity of the

resulting erythropoietin proteins has also been evaluated by commercially available radioimmunoassay protocols.

In particular, the arginine 103 site is essential for erythropoietin activity. As shown herein, replacement of the arginine 103 by another amino acid results in a modified erythropoietin with significantly decreased activity. Modifications at this site, as well as other sites within Domain 1, can similarly be made to enhance regulating activity, as well as to decrease, or reduce regulating ability.

The modified secretable erythropoietin proteins described herein provide useful reagents to further elucidate the structure/function relationship of erythropoietin and its cellular receptor.

Such modified secretable erythropoietin proteins with altered regulating ability can also be used for therapeutic purposes. For example, modified erythropoietin proteins with enhanced activity would be a more potent therapeutic, therefore requiring a lower effective dose or less frequent administration to an individual. Erythropoietin proteins with decreased activity that still retain their structural integrity and bind to their cognate receptor would be useful to decrease growth and differentiation of red blood cell precursors in certain leukemias and polycythemias. Furthermore, an erythropoietin protein that selectively triggers only certain events within the red blood cell precursor cell would be useful in treating various hematological conditions.

Brief Description of the Drawings

Figure 1 is a schematic diagram of the in vitro mutagenesis protocol. WT = wildtype erythropoietin.

Figure 2 depicts the structure of expression vector pSV-2-erythropoietin.

Figure 3 is a graphic representation of the specific activities of nine mutant erythropoietin proteins.

Figure 4 is a graphic representation of the results of monoclonal antibody precipitation of the mutant erythropoietin proteins.

Figure 5 is a graphic representation of the activity of heat-denatured wildtype erythropoietin as measured by radioimmunoassay (□) and the Krystal bioassay (●).

Figure 6A-6B is a graphic representation of the activity of the 103 mutant erythropoietin proteins as measured by radioimmunoassay (□) and the activity of wildtype erythropoietin (●).

Detailed Description of the Invention

The present invention is based on the identification of amino acid residues of the erythropoietin polypeptide which are critical for its biological activity and secretable properties. These sites have been precisely defined through oligonucleotide-directed mutagenesis and used to create mutant human recombinant erythropoietin proteins which are altered by one, or more, amino acid substitutions and thus differ from wildtype erythropoietin.

Identification of Amino Acid Residues of Human Recombinant Erythropoietin Critical for Biological Activity

Previously, anti-peptide antibodies to several hydrophilic domains of the erythropoietin molecule had demonstrated that antibodies to amino acids 99-110 (Domain 1) and 111-129 (Domain 2) block the hormone's biological activity. Binding of the antibody to a portion of the erythropoietin molecule that participated in receptor recognition would block such recognition, thereby neutralizing erythropoietin's biological activity.

(Sytkowski, A. J. and Donahue, K. A., J. Biol. Chem. 262:1161-1165 (1987)).

A series of mutants across the 99-129 region was produced by sequentially replacing three amino acids with Glu-Phe. Mutations in amino acids 120-129 did not change erythropoietin activity. However, mutations in amino acid residues 99-110 caused a profound structural change which inhibited secretion of the mutant erythropoietin after biosynthesis. (Chern, Y., et al., Eur. J. Biochem. 202:225-229 (1991)). To precisely identify the amino acid site, or sites, critical for receptor recognition and biological activity, amino acids 100-109 were studied by alanine scanning mutagenesis, as described in detail in Example 1.

Briefly, human recombinant erythropoietin cDNA (Adamson, J.W., et al., Proc. Natl. Acad. Sci. USA 83:6465-6469 (1986)) was inserted into the Phagemid vector pSELECT (Promega Corp., Madison, WI) which contains two genes for antibiotic resistance. One of these genes, specific for tetracycline resistance is always functional, while the other, specific for ampicillin resistance, has been inactivated. The single-stranded template for the mutagenesis reaction was prepared by growing cultures of bacteria transformed with the Phagemid and infected with a helper phage. The resulting single-stranded DNA was and isolated.

Two oligonucleotides were annealed to this recombinant ssDNA template. The first oligonucleotide was an ampicillin repair oligo designed to convert the vector to ampicillin resistance and the second oligonucleotide was a mutagenic oligo designed to change a portion of the erythropoietin cDNA sequence.

Subsequently, the mutant second strand was synthesized in vitro using T4 DNA polymerase and ligated. This DNA was then transformed into a repair minus strain

of E. coli and these cells were grown in the presence of ampicillin. The phagemid was then harvested and a second round of transformation was carried out and mutants were selected on ampicillin plates. This results in the production of double stranded phagemid containing both the ampicillin resistance gene and the mutated erythropoietin cDNA.

Figure 1 shows the region of the erythropoietin cDNA encoding amino acids 96-113 (SEQ ID NO: 2) and the corresponding wildtype erythropoietin DNA sequence encoding amino acids 96-113 (SEQ ID NO: 3). The column of numbers on the left hand side of Figure 1 indicates the amino acid substitution. The only amino acid residue substitutions made are as indicated. The remainder of the human recombinant erythropoietin DNA sequence was not altered. (The remaining, unaltered human recombinant DNA sequence is not shown.) Thus, for example, 100A (SEQ ID NO: 4) indicates that amino acid 100, normally a serine residue, was replaced by alanine, 101A (SEQ ID NO: 5) indicates that glycine 101 was replaced by alanine, and so forth (SEQ ID NOS: 6-16).

Some sites were mutated more than once. For example, amino acid 103 was mutated twice. The first mutation was the substitution of alanine for arginine 103 (SEQ ID NO: 7) and the second substitution was aspartic acid for arginine (SEQ ID NO: 8).

Two double mutants were also produced, 108A/113R (SEQ ID NO: 12) and 109A/113R (SEQ ID NO: 13). In these two instances, amino acids 108 and 109 were each substituted with alanine in the second mutation and the replacement of glycine 113 with arginine was introduced. The changes in nucleotide sequence in each mutagenic oligo are indicated in Figure 1 and Table I (SEQ ID NOS: 4-22). In Table I, the underlined nucleotides are those which differ from the wildtype erythropoietin sequence. A silent mutation

designed to introduce a restriction site, Hinf I, allowing convenient initial screening for mutated erythropoietin cDNAs, was also introduced.

Each mutated erythropoietin cDNA was identified by restriction analysis, using standard laboratory protocols, and its structure was confirmed by DNA sequencing. The mutated erythropoietin cDNA was then inserted into the expression vector pSV-2 (Figure 2) using standard laboratory techniques. (Mulligan, R. C., et al. Nature 277:108-114 (1979); Maniatis, T., et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratories, New York (1982)).

As described in detail in Example 2, COS-7 cells were transfected with the pSV-2-erythropoietin constructs. After three days, the supernatant medium was harvested and the biological activity of the mutant erythropoietin proteins and wildtype erythropoietin was measured by the Krystal bioassay (Krystal, G., Exp. Hematol. 11:649-660 (1983)). Briefly, the bioassay of Krystal measures the effect of erythropoietin on intact mouse spleen cells. Mice were treated with phenylhydrazine to stimulate production of erythropoietin-responsive red blood cell progenitor cells. After treatment, the spleens were removed, intact spleen cells were carefully isolated and incubated with various amounts of wildtype erythropoietin or the mutant erythropoietin proteins described herein. After an overnight incubation, ^{3}H thymidine was added and its incorporation into cellular DNA was measured. The amount of ^{3}H thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor. The concentration of mutant erythropoietin protein, as well as the concentration of wildtype erythropoietin, was quantified by competitive radioimmunoassay (Incstar, Stillwater, MN). Specific

activities were calculated as international units measured in the Krystal bioassay divided by micrograms as measured as immunoprecipitable protein by RIA. Both assays used wildtype recombinant human erythropoietin standardized against the World Health Organization Second International Reference Standard preparation.

Specific activities of nine of the mutant erythropoietin proteins (SEQ ID NOS: 4-13) described herein are shown in Figure 3. As shown in Figure 3, the specific activities are presented as a percent of the wildtype erythropoietin activity for each mutant erythropoietin. The amino acid replaced by alanine is indicated along the horizontal axis. Table I also shows the specific activities of the nine mutant erythropoietin proteins (SEQ ID NOS: 4-13) as well as nine additional mutant erythropoietin proteins (SEQ ID NOS: 14-22). The specific activity noted in Table I is also that activity relative to wildtype erythropoietin's activity, which is set at 100%.

As shown in Table I, substitution of arginine 103 by alanine (SEQ ID NO: 7), aspartic acid (SEQ ID NO: 8), asparagine (SEQ ID NO: 17), glutamic acid (SEQ ID NO: 18), glutamine (SEQ ID NO: 19), histidine (SEQ ID NO: 20), and leucine (SEQ ID NO: 21) essentially eliminated erythropoietin biological activity. Substitution of lysine for arginine 103 (SEQ ID NO: 22) decreased activity to approximately 10% of wildtype erythropoietin. Substitution of alanine for serine 104 decreased activity to approximately 16% of wildtype erythropoietin (SEQ ID NO: 14). Substitution of alanine for leucine 105 (SEQ ID NO: 9) reduced the activity to approximately 44 percent of wildtype erythropoietin. Substitution of alanine for leucine 108 (SEQ ID NO: 15) reduced the activity to approximately 37% of wildtype erythropoietin. Substitution of alanine for serine 100 (SEQ ID NO: 4) and

glycine 101 (SEQ ID NO: 5) increased the specific activity of the mutant protein.

However, the most striking result is that obtained by substitution of the 103 arginine amino acid residue (SEQ ID NOS: 7, 8, and 17-21). The results of these experiments indicate that arginine 103 is essential for erythropoietin biological activity. Although all of these mutants were expressed and secreted into culture medium at rates equivalent to that seen for wildtype and other mutants, none exhibited any detectable biological activity. Methods described herein, such as the ex vivo bioassay of Krystal (Krystal, G., Exp. Hematol. 11:649-660 (1983)), which is an art-recognized bioassay used to evaluate erythropoietin activity, showed that these arginine 103 mutants are reduced in activity by at least a 1000-fold below that of the wildtype human recombinant erythropoietin.

TABLE I

ALANINE SCANNING MUTAGENESIS OF AMINO ACIDS 100-109 OF ERYTHROPOIETIN

MUTANT	OLIGONUCLEOTIDE	SPECIFIC ACTIVITY	SEQ ID NO:
S100A	GGATAAAGCCGTCGCTGGCCTTCGCAGCCTCACGACTCTGCTTCGGG	107.9%	4
G101A	GCCGTCAGTGCCCTTCGCAGCCTCACGACTCTGCTTCGGG	126.8%	5
L102A	GCCGTCAGTGGCGCTCGCAGCCTCACC	93.3%	6
R103A	CGTCAGTGGCCTTGCCAGCCTCACGACTCTGCTTCGG	0.0%	7
R103D	CGTCAGTGGCCTTGACAGCCTCACGACTCTGCTTCGG	0.0%	8
L105A	GGCCTTCGCAGCGCCACGACTCTGCTTCGGG	44.0%	9
T106A	GCCTTCGCAGCCTCGCGACTCTGCTTCGGGC	76.9%	10
T107A	CGCAGCCTCACCGCTCTGCTTCGAGCTCTGCGAGCC	86.6%	11
L108A/G113R	GCCTCACCACTGCCTTCGAGCTCTGCGAGCC	77.3%	12
L109A/G113R	CCTCACCACTCTGGCTCGGGCTCTGCG	84.7%	13
S104A	GTGGCCTTCGCGCCCTCACGACTCTGCTTC	16.3%	14
L108A	CCTCACCACTGCGCTTCGAGCTCTGGGAGC	36.9%	15
L109A	CCTCACCACTCTGGCTCGGGCTCTGGG	70.2%	16
R103N	CGTCAGTGGCCTTAACAGCCTCACGACTCTGCTTCGG	0.0%	17
R103E	CGTCAGTGGCCTTGAGAGCCTCACGACTCTGCTTCGG	0.0%	18
R103Q	CGTCAGTGGCCTTCAGAGCCTCACGACTCTGCTTCGG	0.0%	19
R103H	CGTCAGTGGCCTTCACAGCCTCACGACTCTGCTTCGG	0.0%	20
R103L	CGTCAGTGGCCTTCTCAGCCTCACGACTCTGCTTCGG	0.0%	21
R103K	CGTCAGTGGCCTGAAGAGCCTCACGACTCTGCTTCGG	10.2%	22

Structural Integrity of Mutant Erythropoietin Proteins

Previously published studies indicated that mutations in the Domain 1 region in which a group of three amino acids was deleted and replaced with Glu-Phe, caused pronounced structural changes in the molecule. (Chern, Y., et al., Eur. J. Biochem. 202:225-229 (1991)). These structural changes were accompanied by lack of secretion of the mutant erythropoietin from the transfected COS-7 cells. Surprisingly, this phenomenon was not observed with the more subtle mutations of the present invention. Thus, the mutant erythropoietin proteins described herein provide structurally intact (i.e., with the proper biological conformation) mutant erythropoietin proteins.

Assessment of the structural integrity of the mutated erythropoietin proteins of the instant invention was performed by a series of immunoprecipitation experiments using anti-peptide monoclonal antibodies to two domains of the protein, as described in Example 3.

Briefly, the first monoclonal antibody recognizes an epitope within amino acids 1-26 of erythropoietin. The other monoclonals recognize distinct epitopes within amino acids 99-129. It is known that a gross change in the tertiary structure of erythropoietin would result in an inability of one or more of the monoclonal antibodies to recognize the erythropoietin molecule. For example, it has been demonstrated that radio-iodination of erythropoietin in the presence of chloramine-T denatures the molecule, resulting in loss of biological activity and corresponding loss of recognition by monoclonal antibody (data not shown).

Figure 4 shows mutant erythropoietin protein precipitated as percent of control of wildtype erythropoietin precipitated using three monoclonal antibodies designated across the horizontal axis, 1-26, 99-129α and 99-129β. The three erythropoietin proteins

examined were the wildtype erythropoietin, the 103 alanine mutant and the 103 aspartic acid mutant. As seen on the left side of the graph, monoclonal 1-26 recognized each of the three recombinant erythropoietin proteins with equal efficiency, indicating that mutation of amino acid 103 to either alanine or aspartic acid did not result in a gross distortion of erythropoietin's conformation.

Similarly, as shown in the center of the graph, monoclonal 99-129α also recognized the wildtype, 103 alanine mutant and 103 aspartic acid mutant with no statistically significant difference among them. This indicates that the conformation within the amino acids 99-129 is similar among the three recombinant erythropoietin proteins.

Lastly, as shown on the right side of the graph, monoclonal 99-129β recognized both mutant erythropoietin proteins with approximately half the efficiency as it recognized the wildtype erythropoietin. This is consistent with the subtle structural change introduced by a single amino acid mutation. Taken together, it is reasonable to assume that the inactive point mutants, 103 alanine and 103 aspartic acid, are not grossly denatured.

Heat Stability of Mutant Erythropoietin Proteins

For studies comparing in vitro biological activity with antibody reactivity, aliquots of conditioned medium from erythropoietin cDNA-transfected COS cells were incubated at 56°C for specified time intervals. The samples were cooled on ice and a fraction of each was assessed for biological activity in the Krystal bioassay. The remainder was split into two fractions and erythropoietin protein was quantified by radioimmunoassay using the commercially available INCSTAR RIA kit. The results are given in terms of the percent biological

activity remaining or percent protein immunoprecipitated after heat treatment compared to untreated samples.

Wildtype erythropoietin exhibits a time-dependent decrease in biological activity when incubated at 56°C or above (Figure 5); Tsuda, E., et al., Eur. J. Biochem. 188:405-411 (1990). Interestingly, a corresponding decrease in the ability of the commercial radioimmunoassay's antibodies to recognize this heat-denatured erythropoietin was also observed (Figure 5). This observation was quite reproducible and enabled the use of the RIA to measure the heat stability of the inactive R103A erythropoietin compared to that of wildtype erythropoietin. As seen in Figure 6A, the heat denaturation curves of R103A and wildtype erythropoietin are essentially identical.

To confirm that this heat stability comparison is sensitive to mutations in this region of erythropoietin, the effect of the aspartic acid substitution (R103D) on the protein's stability was evaluated. The introduction of a negatively charged amino acid residue would reasonably be more structurally disruptive to the molecule than an alanine, and thus be more likely to alter the protein's heat-denaturation curve. The heat stability of R103D was markedly different (i.e., greater) than that of wildtype erythropoietin and R103A, as anticipated (Figure 6B).

To further characterize the nature of the interaction between amino acid residue 103 and the erythropoietin receptor, site-directed mutagenesis was used to produce erythropoietin analogs with altered side chain properties at this position. Arginine was substituted with histidine (R103H), lysine (R103K), asparagine (R103N), glutamine (R103Q), leucine (R103L) and glutamic acid (R103E) to generate 6 new altered erythropoietin molecules. Culture supernatants of cells transfected with these constructs

were tested in the Krystal bioassay and the heat stability assay for biological activity and structural stability, respectively.

Of the six mutants tested, only R103K had detectable levels of biological activity in the Krystal bioassay. Its specific activity (in units per microgram erythropoietin protein) was 10.2% ± 1.3% that of wildtype erythropoietin. The heat denaturation curve of R103K was essentially identical to that generated for the wildtype protein. Interestingly, the heat denaturation curve for R103E was notably different from that of wildtype, and very similar to that of R103D. The other 4 mutants had denaturation kinetics intermediate to that of these two proteins. (Data not shown)

Production of Additional Erythropoietin Proteins Having Altered Biological Activity

As a result of the identification of sites which are critical to erythropoietin activity in terms of the amino acid residue present and which can be altered to produce a mutated sequence which has altered activity but retains its structural integrity, it is now possible to produce modified secretable human recombinant erythropoietin proteins whose ability to regulate the growth and differentiation of red blood cell progenitors is altered (i.e., whose ability to regulate red blood cell progenitors is different from that of the corresponding wildtype human recombinant erythropoietin).

As described in the previous sections and in the Examples, such sites have been identified by oligonucleotide-directed mutagenesis and used to create mutant erythropoietin which resulted in substitution of amino acids at positions 100-109 within Domain 1 (SEQ ID NO: 1), as represented in Figure 1 (SEQ ID NOS: 4-13) and Table I (SEQ ID NOS: 4-16). The data indicate that

arginine 103 is critical for erythropoietin's biological activity. Additionally, serine 104, leucine 105 and leucine 108 appear to play a role, as indicated by the decreased activity of these mutants as measured in the above-described bioassays. Importantly, these mutations do not disrupt the structural integrity of the erythropoietin protein, as evidenced by the fact that the mutated protein is secreted. That is, as the data presented herein indicates, these mutant erythropoietin proteins retain their biological conformation. These results also indicate that Domain 1 amino acids 99-110 very likely participate in receptor recognition and activation.

Moreover, as the data presented herein indicates, some mutant erythropoietin proteins also demonstrate increased heat stability relative to the wildtype erythropoietin, even though the biological activity of the mutant has been significantly decreased.

Substitution of alanine at arginine 103 produced erythropoietin mutants with no detectable erythropoietin activity as measured by standard techniques. Mutations at serine 104, leucine 105 and leucine 108 also significantly decreased activity. In a similar manner, other changes at one or more of these critical sites can result in reduction of erythropoietin activity. Conversely, amino acid residues can be introduced at these critical sites to produce modified secretable human recombinant erythropoietin proteins with enhanced biological activity.

Conservative substitutions can be made at one or more of the amino acid sites within residues 100-109 of the molecule. For example, alanine and aspartic acid have been used to replace arginine 103. Substitution of these amino acids by other amino acids of the same type (i.e., a positively charged, or basic, amino acid for a positively charged, or basic, one, or an acidic amino acid for an

acidic one) as that present at that specific position can be made and the effect on erythropoietin's ability to regulate the growth and differentiation of red blood cell progenitors can be determined, using the methods described herein.

Substitutions at these critical sites, alone or in combination, of amino acids having characteristics different from those of amino acids whose presence at those sites has been shown to eliminate or reduce erythropoietin activity can also be made and their effect on activity assessed as described above. In particular, substitutions of some, or all, of the amino acids at one, or more, of these critical sites which result in modified secretable erythropoietin proteins with enhanced erythropoietin activity can be made. Using the techniques described herein, erythropoietin proteins having enhanced activity can be identified.

In addition, more radical substitutions can be made. For example, an amino acid unlike the residue present in the corresponding position in the wildtype sequence is substituted for the residue in wildtype erythropoietin (e.g., a basic amino acid is substituted for an acidic amino acid). Each resulting mutant is then evaluated using the anti-erythropoietin immunoprecipitation techniques and biological activity assays as described.

As a result, modified secretable human recombinant erythropoietin proteins having enhanced erythropoietin activity or stability can be identified. Similar techniques can be used to identify additional critical sites and subsequently, to make substitutions and evaluate their effects on erythropoietin regulating activity.

Applications of Modified Secretable Erythropoietin Proteins Having Altered Biological Activity

As described above, arginine 103 is essential for erythropoietin's biological activity. Additionally, serine 104, leucine 105 and leucine 108 also appear to play a significant role in biological activity. Furthermore, these subtle point mutations do not compromise the structural integrity, (i.e., secretability) of the erythropoietin molecule. Thus, it is reasonable to assume that the mutant erythropoietin proteins will be recognized by the erythropoietin cellular receptor in essentially the same manner as the wildtype erythropoietin.

Modified secretable human recombinant erythropoietin proteins of the present invention can be used for therapy and diagnosis of various hematologic conditions. For example, an effective amount of modified secretable recombinant erythropoietin with enhanced activity to regulate the growth and differentiation of red blood cell progenitors can be used therapeutically (in vivo) to treat individuals who are anemic (e.g. as a result of renal disease, chemotherapy, radiation therapy, or AIDS). An effective amount of modified secretable human recombinant erythropoietin protein, as defined herein, is that amount of modified secretable erythropoietin protein sufficient to regulate growth and differentiation of red blood cell progenitor cells. For example, modified secretable erythropoietin protein with increased regulatory ability will bind to the erythropoietin receptor and stimulate the growth and differentiation of red blood cell progenitor cells. The modified secretable erythropoietin with enhanced activity would be more potent than the wildtype erythropoietin. Thus, to increase red blood cell growth and differentiation in anemic conditions, a lower

effective dose or less frequent administration to the individual would be required.

Modified secretable erythropoietin with altered regulating activity can also be used to selectively trigger only certain events regarding the growth and differentiation of red blood cell precursors. For example, it has recently been shown that binding of erythropoietin to its receptor generates two distinct chemical signals in cells, a protein kinase C dependent activation of the proto-oncogene c-myc and a phosphatase mediated signal to c-myb. (Spangler, R., et al., J. Biol. Chem. 266:681-684 (1991); Patel, H. R. and Sytkowski, A. J., Abstract 1208, Blood 78(10) Suppl. 1 (1991)). Thus, a modified secretable erythropoietin can be used to selectively activate either the protein kinase C or the phosphatase pathways.

An effective amount of modified secretable erythropoietin with decreased activity, (i.e., reduced activity or no activity), can be used to treat individuals with various erythroleukemias. Thus, in this case, an effective amount of modified secretable erythropoietin protein with decreased regulatory ability will bind to the erythropoietin cellular receptor. However, upon the mutant erythropoietin protein binding to the receptor, the mutant protein lacks ability to trigger subsequent erythropoietin events. Moreover, because the mutant erythropoietic is bound to the receptor, it prevents wildtype erythropoietic from binding to the receptor (i.e., competitively inhibits the binding of wildtype erythropoietin). Thus, the red blood cell progenitors do not proliferate and/or differentiate. The mutant erythropoietin proteins of the present invention are secretable, indicating that they retain their structural integrity, and thus fully participate in receptor recognition and binding. The initial interaction of a

hormone with its cognate receptor might be expected to result in further conformational changes of the hormone ligand, thereby stabilizing the hormone/receptor complex and allowing the formation of higher ordered complexes.

However, if a modified erythropoietin protein of the present invention, with no detectable erythropoietin activity, binds to its receptor, it is reasonable to assume that the subsequent events triggered by receptor binding will be altered or inhibited. Therefore, it is also reasonable to assume that growth and differentiation of red blood cell progenitor cells will be altered or inhibited, thereby inducing a remission in a red blood cell leukemia.

Recently, a constitutively active (hormone independent) form of the murine erythropoietin receptor was isolated. (Watowich, S. S., Proc. Natl. Acad. Sci. USA 89:2140-2144 (March 1992). It has also been shown that the envelope glycoproteins of certain murine viruses bind to and activate the murine erythropoietin receptor. (Yoshimura, A. Proc. Natl. Acad. Sci. USA 87:4139-4143 (June 1990)). Thus, erythropoietin-independent activation (constitutive activation) of the erythropoietin receptor resulting in red blood cell proliferation in a mammal has been demonstrated. It is reasonable to predict that similar constitutive activation would occur in humans, (for example, a virus similar to Rauscher or Friend virus) may constitutively activate the human erythropoietin receptor also resulting in proliferation of red blood cell progenitors. A modified secretable erythropoietin, which retains its structural integrity to bind to the receptor, yet does not activate red blood cell proliferation, would be useful as an antagonist to block such constitutive activation. Moreover, modified secretable erythropoietin proteins with increased stability would provide long-acting erythropoietin antagonists.

Modified secretable erythropoietin would be useful to treat various hemoglobinopathies and hemolytic anemias. For example, polycythemia vera is characterized by uncontrollable proliferation of red blood cells and is currently treated by chemotherapy, radiation or phlebotomy. The increased number of red blood cells increases blood viscosity, leading to a hypertensive condition that can result in a stroke. It is reasonable to predict that an antagonist of erythropoietin, which binds to the receptor and blocks activation, would be a useful, non-invasive treatment.

Likewise, individuals with cyanotic heart disease often have a hyper-erythropoietin condition, leading to increased erythrocyte proliferation. Also, renal disease patients that are being treated with wildtype erythropoietin may experience an overdose. Once the wildtype erythropoietin has been administered, it continues to act. Thus, in these cases, it would be useful to administer a modified secretable erythropoietin with decreased activity to block the effects of the endogenous and exogenous erythropoietin.

Furthermore, certain hemolytic anemias, such as sickle cell anemia and thalassemia, result in rapid destruction of red blood cells. The body responds by increasing the levels of erythropoietin produced to stimulate red blood cell production. However, the red blood cells produced carry defective hemoglobin. It would be useful to use a modified secretable erythropoietin to reduce production of defective erythrocytes while another form of therapy is used to stimulate normal hemoglobin synthesis.

Modified secretable erythropoietin may be administered to individuals parenterally or orally. The modified secretable erythropoietin proteins of this invention can be employed in admixture with conventional

pharmaceutically acceptable carriers. Suitable pharmaceutical carriers include, but are not limited to, water, salt solutions and other physiologically compatible solutions. The modified secretable erythropoietin proteins of the present invention may be administered alone, or combined with other therapeutic agents.

It will be appreciated that the amount of modified secretable erythropoietin administered to an individual in a specific case will vary according to the specific modified secretable erythropoietin protein being utilized, the particular compositions formulated, and the mode of application. Dosages for a given individual can be determined using conventional considerations such as the severity of the condition, body weight, age and overall health of the individual.

Modified secretable erythropoietin can also be used for diagnostic purposes. For example, it can be used in assay procedures for detecting the presence and determining the quantity, if desired, of erythropoietin receptor. A modified secretable erythropoietin with enhanced activity would be useful to increase the sensitivity and decrease the incubation times of such assays. It can also be used in in vitro binding assays to determine the effect of new drugs on the binding of erythropoietin protein to its receptor.

Modified secretable erythropoietin proteins described herein also provide useful research reagents to further elucidate the role of erythropoietin in erythropoiesis, as well as the structure/function relationship of erythropoietin and its cellular receptor.

This invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLE 1 Oligonucleotide-directed Mutagenesis of Human Recombinant Erythropoietin

The oligonucleotide-directed mutagenesis used to prepare the modified secretable human recombinant erythropoietin proteins of the present invention was performed using the Altered Sites™ In Vitro Mutagenesis System (Promega Corporation of Madison, WI). The Altered Sites™ system consists of a unique mutagenesis vector and a simple, straightforward procedure for selection of oligonucleotide-directed mutants. The system is based on the use of a second mutagenic oligonucleotide to confer antibiotic resistance to the mutant DNA strand. The system employs a phagemid vector, pSELECT™-1, which contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is always functional. The other, for ampicillin resistance, has been inactivated. An oligonucleotide is provided which restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide is annealed to the single-stranded DNA (ssDNA) template at the same time as the mutagenic oligonucleotide and subsequent synthesis and ligation of the mutant strand links the two. The DNA is transformed into a repair minus strain E. coli, or other suitable host, and the cells are grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109, or a similar host, ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants.

The pSELECT-1 plasmid is a phagemid, defined as a chimeric plasmid containing the origin of a single-stranded DNA bacteriophage. This phagemid produces ssDNA upon infection of the host cells with the helper phage R408 or M13KO7. The vector contains a multiple cloning site flanked by the SP6 and T7 RNA polymerase promoters

and inserted into the lacZ α-peptide. Cloning of a DNA insert into the multiple cloning site results in inactivation of the α-peptide. When plated on indicator plates, colonies containing recombinant plasmids are white in a background of blue colonies. The SP6 and T7 promoters may be used to generate high specific activity RNA probes from either strand of the insert DNA. These sites also serve as convenient priming sites for sequencing of the insert. The pSELECT-1 vector carriers gene sequences for both ampicillin and tetracycline resistance. However, the plasmid is ampicillin sensitive because a frameshift was introduced into this resistance gene by removing the Pst I site. Therefore, propagation of the plasmid and recombinants is performed under tetracycline selection.

The pSELECT-Control vector provides a convenient white/blue positive control for mutagenesis reactions. This vector was derived from the pSELECT-1 vector by removing the Pst I site within the polylinker. The resultant frameshift in the lac α-peptide inactivated β-galactosidase and led to a white colony phenotype on indicator plates. A lacZ repair oligonucleotide (supplied with the system) may be used to introduce a four base insertion which corrects the defect in the lacZ gene and restores colony color to blue. The fraction of blue colonies obtained is an indication of the mutagenesis efficiency. When the lacZ repair oligonucleotide is used in combination with the ampicillin repair oligonucleotide to correct this defect, 80-90% of the ampicillin resistant colonies are blue. When the lacZ repair oligonucleotide is used alone, a mutagenesis efficiency of only 2-5% is seen.

The mutagenic oligonucleotide must be complementary to the single-stranded target DNA. The ssDNA produced by

the pSELECT-1 phagemid is complementary to the lacZ coding strand.

The stability of the complex between the oligonucleotide and the template is determined by the base composition of the oligonucleotide and the conditions under which it is annealed. In general, a 17-20 base oligonucleotide with the mismatch located in the center will be sufficient for single base mutations. This gives 8-10 perfectly matched nucleotides on either side of the mismatch. For mutations involving two or more mismatches, oligonucleotides of 25 bases or longer are needed to allow for 12-15 perfectly matched nucleotides on either side of the mismatch.

Routinely, oligonucleotides can be annealed by heating to 70°C for 5 minutes followed by slow cooling to room temperature.

DNA to be mutated is cloned into the pSELECT-1 vector using the multiple cloning sites. The vector DNA is then transformed into competent cells of JM109, or a similar host, and recombinant colonies are selected by plating on LB plates containing 15μg/ml tetracycline, 0.5mM IPTG, and 40μg/ml X-Gal. After incubation for 24 hours at 37°C, colonies containing recombinant plasmids will appear white in a background of blue colonies.

To produce single-stranded template for the mutagenesis reaction, individual colonies containing pSELECT-Control or recombinant pSELECT-1 phagemids are grown and the cultures are infected with helper phage as described below. The single-stranded DNA produced is complementary to the lacZ coding strand and complementary to the strand of the multiple cloning site. Two helper phages R408 and M13KO7 can be used to provide the greatest latitude in optimizing ssDNA yields.

PROTOCOL

1. Prepare an overnight culture of cells containing pSELECT™-1 or pSELECT™-Control phagemid DNA by picking individual tetracycline resistant colonies from a fresh plate. Inoculate 1-2ml of TYP broth (Promega) containing 15μg/ml tetracycline and shake at 37°C.

2. The next morning inoculate 5ml of TYP broth containing 15μg/ml tetracycline with 100μl of the overnight culture. Shake vigorously at 37°C for 30 minutes in a 50ml tube.

3. Infect the culture with helper phage R408 or M13KO7 at an m.o.i. (multiplicity of infection) of 10 (i.e., add 10 helper phage particles per cell). For the helper phages supplied with this system, add 40μl. Continue shaking for 6 hours to overnight with vigorous agitation.

4. Harvest the culture supernatant by pelleting the cells at 12,000 x g for 15 minutes. Pour the supernatant into a fresh tube and spin again for 15 minutes.

5. Precipitate the phage by adding 0.25 volume of phage precipitation solution (Promega) to the supernatant. Leave on ice for 30 minutes, then centrifuge for 15 minutes at 12,000 x g. Thoroughly drain the supernatant.

6. Resuspend the pellet in 400μl of TE buffer (Promega) and transfer the sample to a microcentrifuge tube.

7. Add 0.4ml of chloroform:isoamyl alcohol (24:1) to lyse the phage, vortex for 1 full minute, and centrifuge in a microcentrifuge (12,000 x g) for 5 minutes. This step removes excess PEG.

8. Transfer the upper, aqueous phase (containing phagemid DNA) to a fresh tube, leaving the interface behind. Add 0.4ml of TE-saturated phenol:chloroform to the aqueous phase, vortex for 1 full minute, and centrifuge as in step 7.

9. Transfer the upper, aqueous phase to a fresh tube and repeat the phenol extraction as in step 8. If necessary, repeat this extraction several times until there is no visible material at the interface.

10. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume (200µl) of 7.5M ammonium acetate plus 2 volumes (1.2ml) of ethanol. Mix and leave at -20°C for 30 minutes to precipitate the phagemid DNA.

11. Centrifuge at 12,000 x g for 5 minutes, remove the supernatant, carefully rinse the pellet with 70% ethanol, and centrifuge again for 2 minutes. Drain the tube and dry the pellet under vacuum. The pellet may be difficult to see.

12. Resuspend the DNA in 20µl of H_2O. The amount of DNA present can be estimated by agarose gel electrophoresis of a 2µl sample.

The mutagenesis reaction involves annealing of the ampicillin repair oligonucleotide and the mutagenic oligonucleotide to the ssDNA template, followed by the synthesis of the mutant strand with T4 DNA polymerase.

The heteroduplex DNA is then transformed into the repair minus E. coli strain DMH71-18 mutS or other suitable strain. Mutants are selected by overnight growth in the presence of ampicillin. Plasmid DNA is the isolated and transformed into the JM109 strain, or other suitable strain. Mutant, ampicillin resistant colonies may be screened by direct sequencing of the plasmid DNA.

A. Annealing Reaction and Mutant Strand Synthesis

The amount of oligonucleotide required in this reaction may vary depending on the size and amount of the single-stranded DNA template. The ampicillin repair oligonucleotide (27 bases long) should be used at a 5:1 oligo:template ratio and the mutagenic oligonucleotide should be used at a 25:1 oligo:template ratio. A typical reaction may contain approximately 100ng (0.05 pmol) of ssDNA.

PROTOCOL

1. Prepare the mutagenesis or control annealing reactions as described below.

Mutagenesis Annealing Reaction

Recombinant pSELECT™-1 ssDNA	0.05pmol
Ampicillin repair oligonucleotide (2.2ng/μl)	1μ(0.25pmol)
Mutagenic oligonucleotide, phosphorylated (see Table 1)	1.25 pmol
10X Annealing buffer	2μl
Sterile H_2O	to final volume 20μl

Control Annealing Reaction

pSELECT™-Control ssDNA	100ng(0.05pmol)
Ampicillin repair oligonucleotide (2.2ng/μl)	1μl(0.25pmol)
lacZ control oligonucleotide (10.8ng/μl)	1μl(1.25pmol)
10X Annealing buffer	2μl
Sterile H_2O	to final volume 20μl

2. Heat the annealing reaction to 70°C for 5 minutes and allow it to cool slowly to room temperature (15-20 minutes).

3. Place the annealing reaction on ice and add the following:

10X Synthesis buffer	3μl
T4 DNA polymerase (10u/μl)	1μl
T4 DNA ligase (2u/μl)	1μl
Sterile H_2O	5μl
	to final volume 20μl

4. Incubate the reaction at 37°C for 90 minutes to perform mutant strand synthesis and ligation.

Table 1. Amount of Mutagenic Oligonucleotide Needed to Equal 1.25 pmol.

Primer Length	ng of Primer Equal to 1.25pmol
17mer	7.0ng
20mer	8.3ng
23mer	9.5ng
26mer	10.8ng
29mer	12.0ng

B. Transformation into BMH 71-18 mutS

PROTOCOL

1. Add 3µl of DMSO to 200µl of BMH71-18 mut S competent cells, mix briefly, and then add the entire synthesis reaction from step A.4.

2. Let the cells sit on ice for 30 minutes.

3. OPTIONAL: For some strains, a heat shock at 42°C for 1-2 minutes after the incubation on ice has been reported to increase transformation efficiency. In our experience, however, a heat shock does not significantly affect the efficiency of transforming BMH71-18 mut S.

4. Add 4ml of LB medium and incubate at 37°C for 1 hour to allow the cells to recover.

5. Add ampicillin to a final concentration of 125µg/ml and incubate at 37°C for 12-14 hours with shaking.

NOTE: As a control to check the synthesis reaction, 1 ml of the culture can be removed after the one hour recovery step, spun down, resuspended in 50µl of LB medium, and plated on LB plates (pg. 12) containing 125µg/ml ampicillin. This is a check for the presence of ampicillin resistant transformants; a second round of transformation is necessary before screening for mutants.

C. Plasmid Mini-Prep Procedure

This procedure is used to isolate pSELECT-1 or pSELECT-Control plasmid DNA from the overnight culture of BMH 71-18 mut S (step B.5, above). A yield of 1-3µg of plasmid DNA may be expected.

PROTOCOL

1. Place 1.5ml of the overnight culture into a microcentrifuge tube and centrifuge at 12, 000 x g for 1 minute. The remainder of the overnight culture can be stored at 4°C.

2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

3. Resuspend the pellet by vortexing in 100µl of ice-cold miniprep lysis buffer (Promega).

4. Incubate for 5 minutes at room temperature.

5. Add 200 µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS. Mix by inversion. DO NOT VORTEX. Incubate for 5 minutes on ice.

6. Add 150µl of ice-cold potassium acetate solution, pH 4.8 (Promega). Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.

7. Centrifuge at 12,000 x g for 5 minutes.

8. Transfer the supernatant to a fresh tube, avoiding the white precipitate.

9. Add 1 volume of TE-saturated phenol/chloroform (Promega). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

10. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol)24:1). Vortex for 1 minute and centrifuge as in step 9.

11. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of 100% ethanol. Mix and allow to precipitate 5 minutes on dry ice.

12. Centrifuge at 12,000 x g for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.

13. Dissolve the pellet in 50μl of sterile deionized water. Add 0.5μl of 100μg/ml DNase-free RNase A (Promega) and incubate for 5 minutes at room temperature.

14. The yield of plasmid DNA can be determined by electrophoresis on an agarose gel.

D. Transformation into JM109 Host Cells

PROTOCOL

1. Add 3μl of DMSO to 200μl of JM109 competent cells, mix briefly, and add 0.05-0.10μg of plasmid DNA from step C.14. Other suitable host cells may be used.

2. Let the cells sit on ice for 30 minutes.

3. OPTIONAL: A heat shock may be performed at this step.

4. Add 2ml of LB medium and incubate at 37°C for 1 hour to allow the cells to recover.

5. Divide the culture into two microcentrifuge tubes and spin for 1 minute in a microcentrifuge.

6. Pour off the supernatant and resuspended the cells in each tube in 50μl of LB medium.

7. Plate the cells in each tube on an LB plate containing 125μg/ml ampicillin and incubate at 37°C for 12-14 hours.

E. Analysis of Transformants

The Altered Sites mutagenesis procedure generally produces greater than 50% mutants, so colonies may be screened by direct sequencing. A good strategy is to pick 10 colonies and start by sequencing 4 of these. If the mutation is located within 200-300 bases of either end of the DNA insert, the SP6 or T7 sequencing primers may be used for convenient priming of the sequencing reactions.

EXAMPLE 2 Cell culture and Transfection

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO). Transient expression of cDNAs was performed using a DEAE-Dextran protocol modified by 0.1mM chloroquine treatment (Sussman, D.J. & Milman, Mol. Cell Biol. 4:1641-1645 (1984); Ausubel, F.M., et al., "Current Protocols in Molecular Biology" pp.921-926, John Wiley and Son, New York, (1989)). 3 days before the transfection, COS-7 cells were plated at 2 x 10^5/10-cm tissue culture dish. 4μg DNA were used in each transfection. Medium was collected 3 days after transfection and assayed for erythropoietin activity and protein.

EXAMPLE 3 Immunoprecipitation of erythropoietin

Wildtype and mutant erythropoietin contained in supernatant medium from COS cell transfections were diluted one- to four-fold with Dulbecco's modified Eagle medium containing 10% fetal bovine serum. After one hour incubation at 37 degrees C with a monoclonal anti-peptide antibody to erythropoietin directed against amino acids 1-26 or 99-129, an equal volume of Omnisorb (Calbiochem) was added to the samples and the suspension was incubated for one hour at 4 degrees C. The Omnisorb was pelleted by centrifugation at 4000 rpm for 30 seconds. The erythropoietin remaining in the supernatant which was not bound by the monoclonal antibody was measured by radioimmunoassay. The amount of erythropoietin bound by antibody (as a percent) was calculated by subtracting the amount in the supernatant from 100%, the starting concentration.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. such equivalents are intended to be encompassed in the scope of the following claims.

Statement Regarding Content of the Sequence Listing in Paper Form and Computer Readable Form

Applicants' Attorney hereby states that the contents of the Sequence Listing in paper form and in computer readable form are the same.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: New England Deaconess Hospital

(ii) TITLE OF INVENTION: RECOMBINANT HUMAN ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/920,810
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Granahan, Patricia
(B) REGISTRATION NUMBER: 32,227
(C) REFERENCE/DOCKET NUMBER: NEDH92-04A PCT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Lys Thr Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala
1 5 10 15

Leu Gly

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGATAAAGCC GTCAGTGGCC TTCGCAGCCT CACCACTCTG CTTCGGGCTC TGGGAGCC 58

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGATAAAGCC GTCGCTGGCC TTCGCAGCCT CACGACTCTG CTTCGGG 47

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCGTCAGTG CCCTTCGCAG CCTCACGACT CTGCTTCGGG 40

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCGTCAGTG GCGCTCGCAG CCTCACC 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGTCAGTGGC CTTGCCAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCATACCAT AGCGTCAGTG GCCTTGACAG CCTCACGACT CTGCTTCGG 49

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCCTTCGCA GCGCCACGAC TCTGCTTCGG G 31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCCTTCGCAG CCTCGCGACT CTGCTTCGGG C 31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCAGCCTCA CCGCTCTGCT TCGAGCTCTG CGAGCCCCTC ACCACTGCGC TTCGAGCTCT 60

GGGAGC 66

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCTCACCAC TGCGCTTCGA GCTCTGCGAG CCCCTCACCA CTCTGGCTCG GGCTCTGGG 59

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCTCACCACT CTGGCTCGGG CTCTGCG 27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGGCCTTCG CGCCCTCACG ACTCTGCTTC 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCTCACCACT GCGCTTCGAG CTCTGGGAGC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCTCACCACT CTGGCTCGGG CTCTGGG 27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGTCAGTGGC CTTAACAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGTCAGTGGC CTTGAGAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGTCAGTGGC CTTCAGAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGTCAGTGGC CTGCACAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGTCAGTGGC CTTCTCAGCC TCACGACTCT GCTTCGG 37

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGTCAGTGGC CTTAAGAGCC TCACGACTCT GCTTCGG 37

CLAIMS

The invention claimed is:

1. DNA encoding modified secretable human recombinant erythropoietin protein which has at least one amino acid residue in Domain 1 which differs from the amino acid residue present in the corresponding position of wildtype erythropoietin and has altered ability to regulate the growth and differentiation of red blood cell progenitor cells.

2. The DNA of Claim 1 encoding a modified secretable human recombinant erythropoietin protein which has decreased ability to regulate the growth and differentiation of red blood cell progenitor cells.

3. The DNA of Claim 1 encoding a modified secretable human recombinant erythropoietin protein which has enhanced ability to regulate the growth and differentiation of red blood cell progenitors.

4. DNA comprising nucleotide triplets encoding modified secretable human recombinant erythropoietin wherein a nucleotide triplet encoding an amino acid residue present in wildtype recombinant erythropoietin amino acid residues 99-110 differs in at least one nucleotide from that present in wildtype erythropoietin and encodes an amino acid other than the amino acid present in the equivalent position in wildtype recombinant erythropoietin.

5. Modified, secretable human recombinant erythropoietin encoded by the DNA of Claim 4.

6. DNA comprising nucleotide triplets encoding modified secretable human recombinant erythropoietin wherein a nucleotide triplet differs from the nucleotide triplet present in the equivalent position in DNA encoding recombinant wildtype erythropoietin the, nucleotide triplet selected from the group consisting of: amino acid residue 103, amino acid residue 104, amino acid residue 105 and amino acid residue 108.

7. Modified secretable human recombinant erythropoietin encoded by the DNA of Claim 6.

8. Modified secretable human recombinant erythropoietin protein which has at least one amino acid residue in Domain 1 which differs from the amino acid residue present in the corresponding position of wildtype erythropoietin and has altered ability to regulate the growth and differentiation of red blood cell progenitors.

9. A modified secretable human recombinant erythropoietin protein of Claim 8 having decreased ability to regulate the growth and differentiation of red blood cell progenitor cells.

10. A modified secretable human recombinant erythropoietin protein of Claim 8 having enhanced ability to regulate the growth and differentiation of red blood cell progenitor cells.

11. A modified secretable human recombinant erythropoietin of Claim 8 having decreased ability to regulate growth and differentiation of red blood cell progenitor cells in which the 103 arginine amino acid residue is substituted with an amino acid residue selected from the group consisting of alanine, aspartic acid, asparagine, glutamic acid, glutamine, histidine, leucine and lysine.

12. A modified secretable human recombinant erythropoietin of Claim 8 having decreased ability to regulate growth and differentiation of red blood cell progenitor cells in which the 104 serine amino acid residue is substituted with the amino acid residue alanine.

13. A modified secretable human recombinant erythropoietin of Claim 8 having decreased ability to regulate growth and differentiation of red blood cell progenitor cells in which the 105 leucine amino acid residue is substituted with the amino acid residue alanine.

14. A modified secretable human recombinant erythropoietin of Claim 8 having decreased ability to regulate growth and differentiation of red blood cell progenitor cells in which the 108 leucine amino acid residue is substituted with the amino acid residue alanine.

15. A modified secretable human recombinant erythropoietin with increased heat stability.

16. A modified secretable human recombinant erythropoietin of Claim 15 wherein the 103 arginine amino acid residue is substituted with an amino acid residue selected from the group consisting of aspartic acid and glutamic acid.

17. A method of decreasing growth and differentiation of red blood cell progenitor cells in an individual, comprising administering to the individual an effective amount of a modified secretable human recombinant erythropoietin protein having decreased ability to regulate the growth and differentiation of red blood cell progenitor cells.

18. The method of Claim 17 wherein the modified secretable human recombinant erythropoietin protein is selected from the group consisting of: modified secretable human recombinant erythropoietin protein in which the 103 arginine amino acid residue is substituted with an amino acid residue selected from the group consisting of alanine, aspartic acid, asparagine, glutamic acid, glutamine, histidine, leucine and lysine.

19. The method of Claim 17 wherein the modified secretable human recombinant erythropoietin protein has the 104 serine amino acid residue substituted with the amino acid residue alanine.

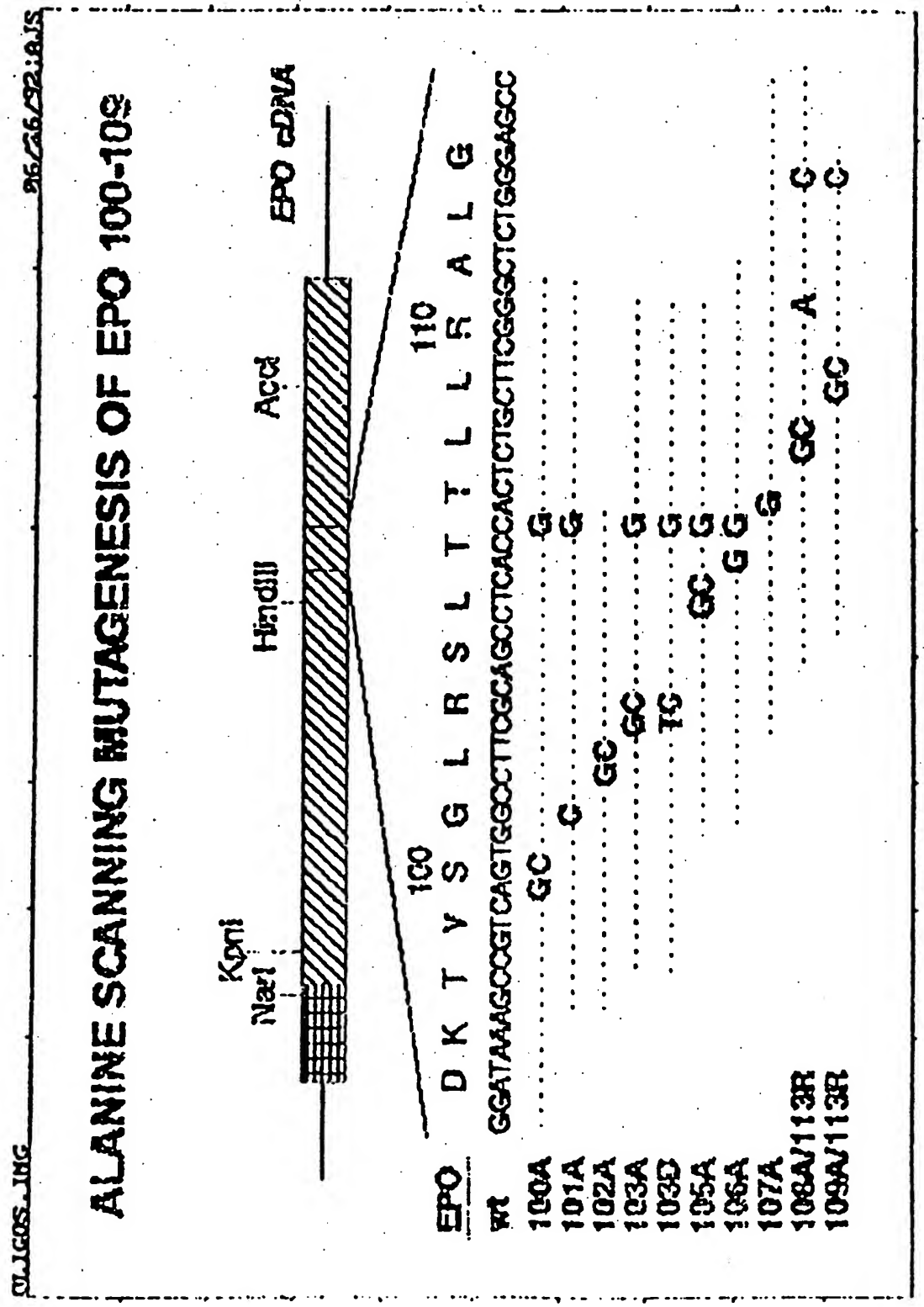
20. The method of Claim 17 wherein the modified secretable human recombinant erythropoietin protein has the 105 leucine amino acid residue substituted with the amino acid residue alanine.

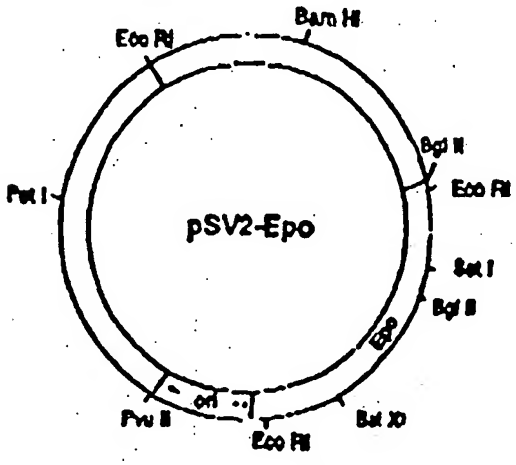
21. A method of enhancing growth and differentiation of red blood cell progenitors in an individual comprising administering an amount of a modified secretable human recombinant erythropoietin protein having enhanced ability to regulate the growth and differentiation of red blood cell progenitors, said amount sufficient to enhance growth and differentiation of red blood cell progenitors.

22. Modified secretable human recombinant erythropoietin protein according to any one of Claims 8, 11, 12, 13 and 14 for use in therapy e.g., enhancing or decreasing growth and differentiation of red blood cell progenitors in an individual.

23. Use of a modified secretable human recombinant erythropoietin protein for the manufacture of a medicament for enhancing or decreasing growth and differentiation of red blood cell progenitors in an individual.

FIGURE 1


OLIGOS.ING
96/26/92:0JS
ALANINE SCANNING MUTAGENESIS OF EPO 100-109
Kpni
NarI
HindIII
AccI
EPO cDNA
100
110
EPO D K T V S G L R S L T T L L R A L G
wt GGATAAAGCCGTCAGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGGGAGCC
100A GC G
101A C G
102A GC
103A GC G
103D TC G
105A GC G
106A G G
107A G
108A/113R GC A C
109A/113R GC C



Structure of expression vector pSV2-Epo

Figure 2

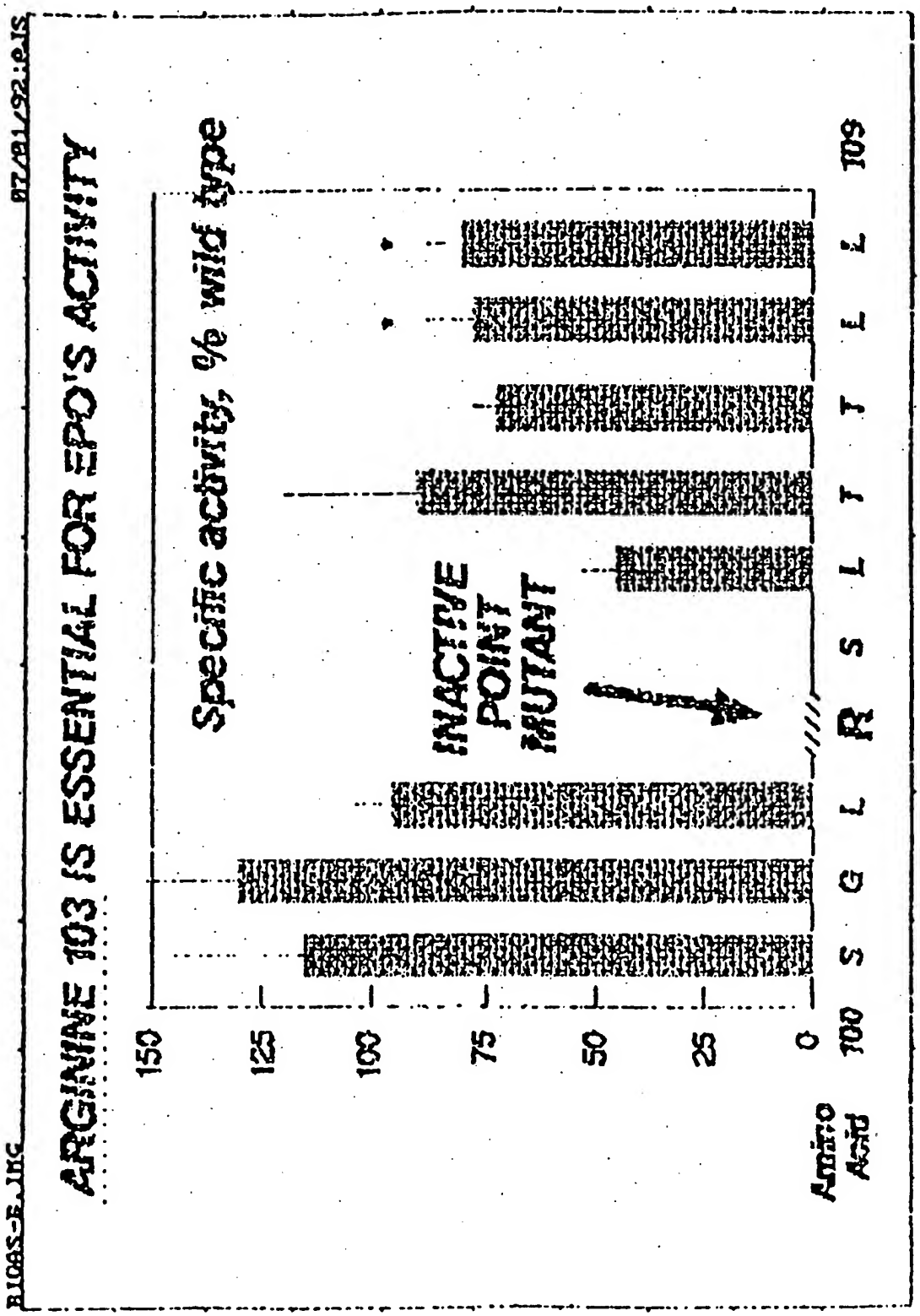

07/01/92:eJS
BIOGEN-BJMC
ARGININE 103 IS ESSENTIAL FOR EPO'S ACTIVITY
Specific activity, % wild type
150
125
100
75
50
25
0
INACTIVE POINT MUTANT
Amino Acid
100 S G L R S L T L L 109

Figure 3

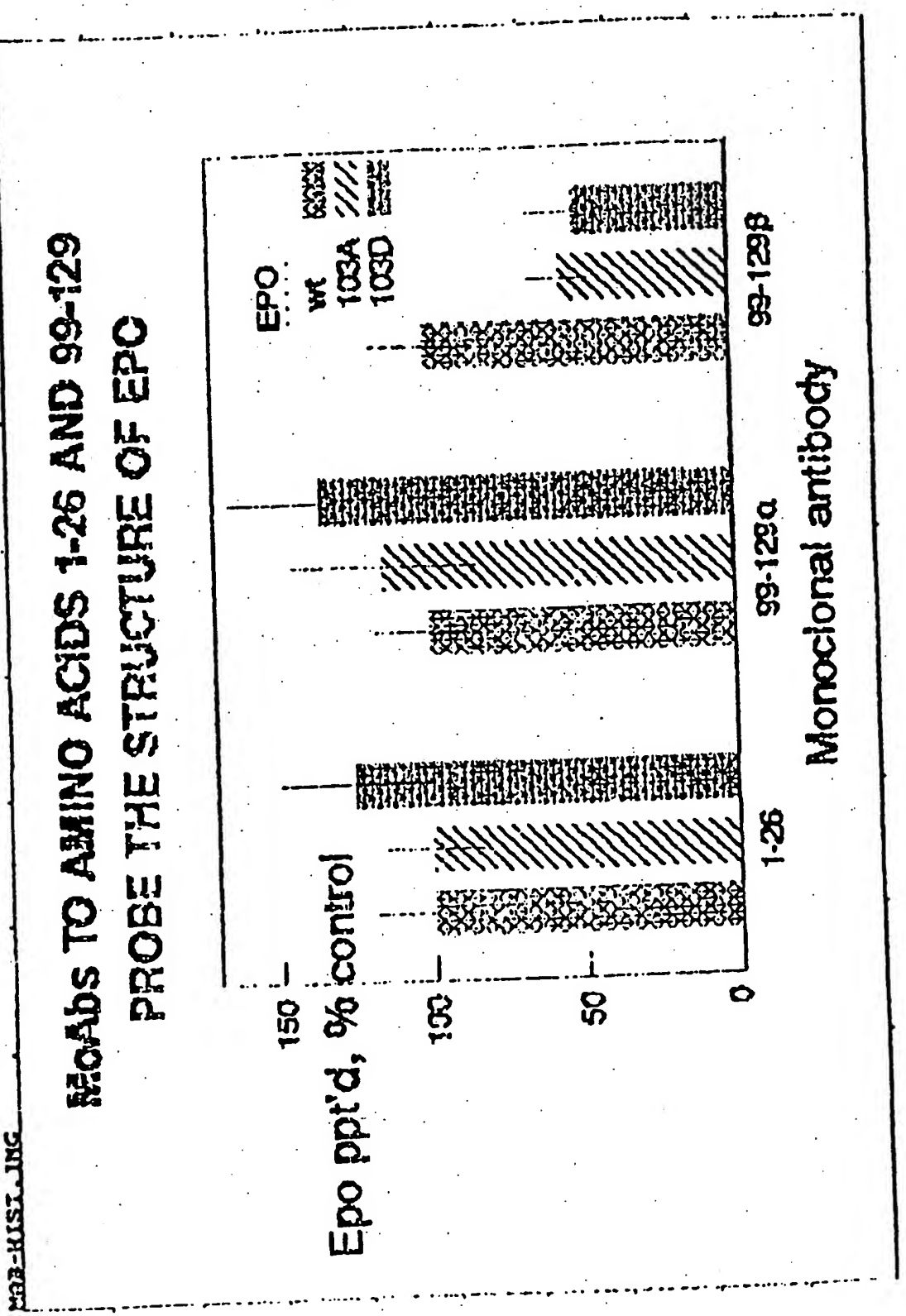

06/26/92:8:15
MAB-HIST.JNG
MoAbs TO AMINO ACIDS 1-26 AND 99-129
PROBE THE STRUCTURE OF EPO
EPO
wt
103A
103D
Epo ppt'd, % control
150
100
50
0
1-26
99-129a
99-129β
Monoclonal antibody

FIGURE 4

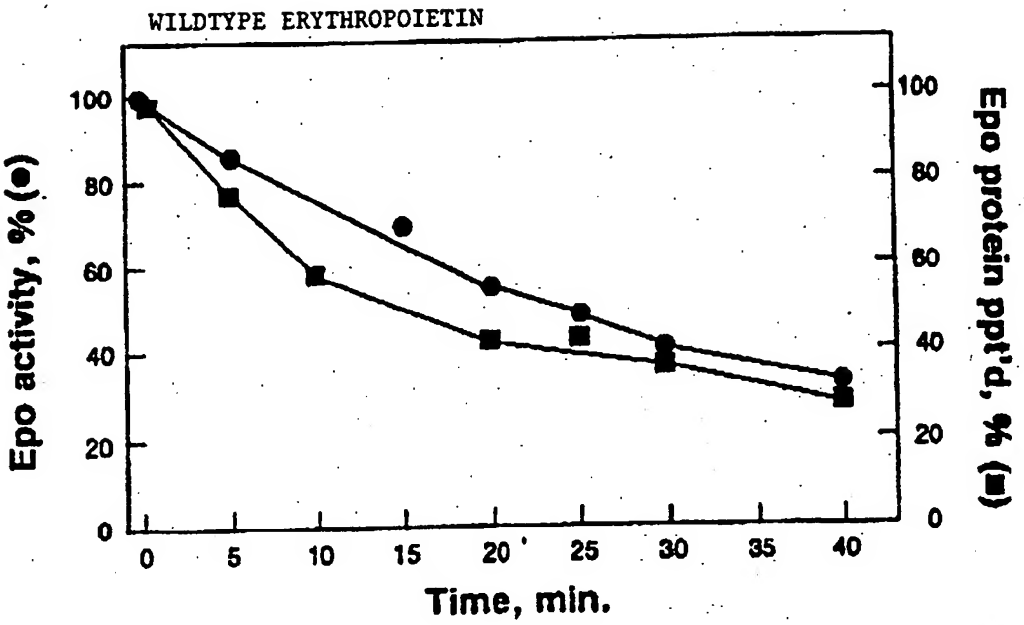

WILDTYPE ERYTHROPOIETIN
Epo activity, % (●)
Epo protein ppt'd, % (■)
Time, min.
0
20
40
60
80
100
0
5
10
15
20
25
30
35
40

FIGURE 5

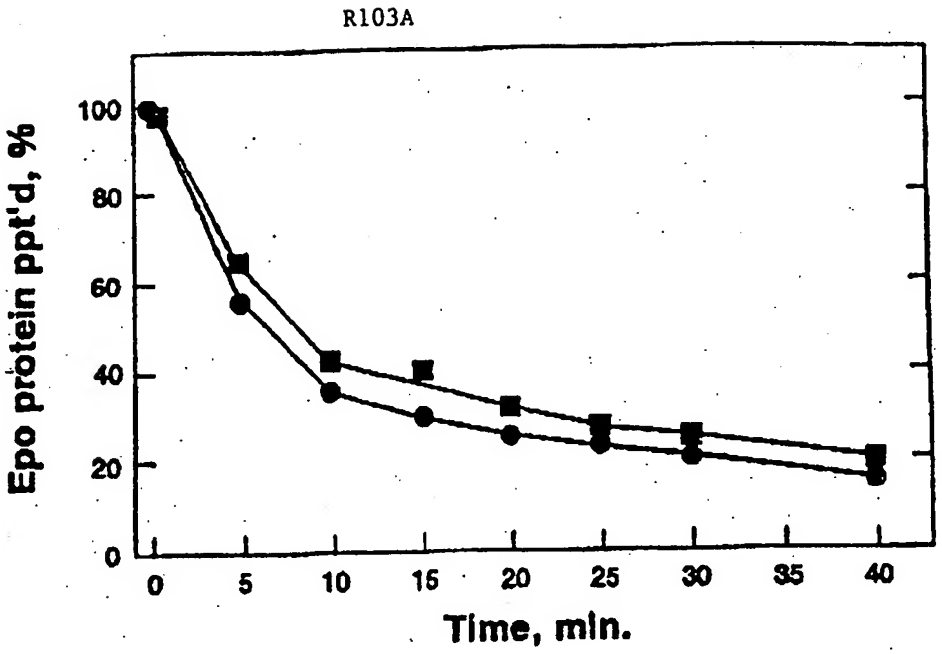


FIGURE 6A

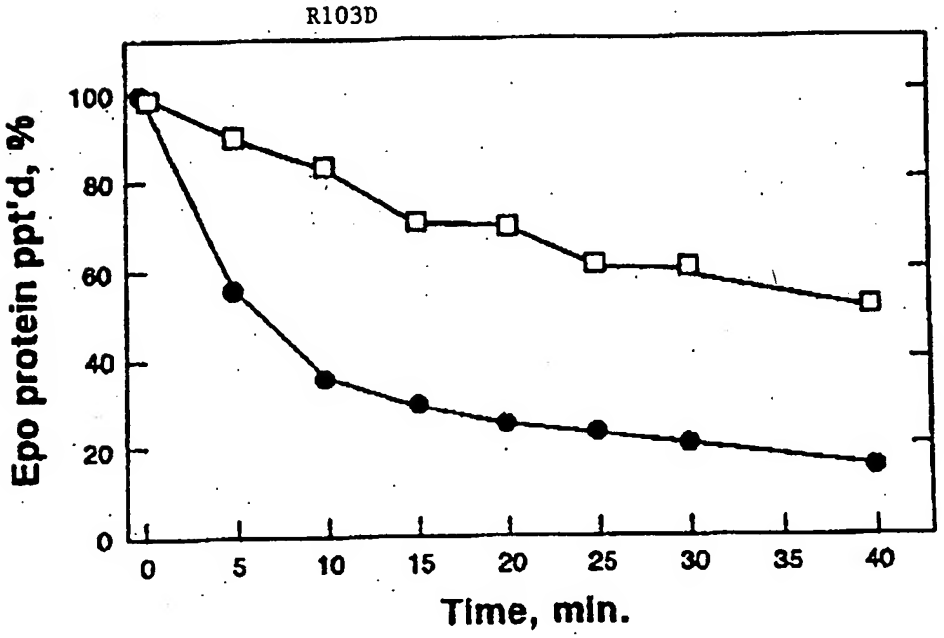


FIGURE 6B